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Lipase Expression in *Pseudomonas alcaligenes* Is Under the Control of a Two-Component Regulatory System▽

Joanna Krzeslak,[1] Gijs Gerritse,[2] Ronald van Merkerk,[1] Robbert H. Cool,[1] and Wim J. Quax[1,2]*

Pharmaceutical Biology, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen,[1] and Genencor International B.V., P.O. Box 218, 2300 AE Leiden,[2] The Netherlands

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Preliminary observations in a large-scale fermentation process suggested that the lipase expression of *Pseudomonas alcaligenes* can be switched on by the addition of certain medium components, such as soybean oil. In an attempt to elucidate the mechanism of induction of lipase expression, we have set up a search method for genes controlling lipase expression by use of a cosmid library containing fragments of *P. alcaligenes* genomic DNA. A screen for lipase hyperproduction resulted in the selection of multiple transformants, of which the best-producing strains comprised cosmids that shared an overlapping genomic fragment. Within this fragment, two previously unidentified genes were found and named *lipQ* and *lipR*. Their encoded proteins belong to the NtrBC family of regulators that regulate gene expression via binding to a specific upstream activator sequence (UAS). Such an NtrC-like UAS was identified in a previous study in the *P. alcaligenes* lipase promoter, strongly suggesting that LipR acts as a positive regulator of lipase expression. The regulating role could be confirmed by down-regulated lipase expression in a strain with an inactivated *lipR* gene and a threefold increase in lipase yield in a large-scale fermentation when expressing the *lipQR* operon from the multicopy plasmid pLAFR3. Finally, cell extracts of a LipR-overexpressing strain caused a retardation of the lipase promoter fragment in a band shift assay. Our results indicate that lipase expression in *Pseudomonas alcaligenes* is under the control of the LipQR two-component system.

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are produced by a wide variety of living organisms. Most microbial lipases are secreted into the extracellular medium, which make them easily recoverable for use in industrial applications. Of particular interest are lipases made by *Pseudomonas* species that have properties compatible with use in household detergents (32), synthesis of pharmaceuticals or agrochemicals (29, 57), and processing of fats (24, 60).

The production of lipases by fermentation, however, is complicated and poorly understood. Some processes have been developed empirically, but the control of lipase synthesis and the reproducibility of the processes are unsatisfactory. Furthermore, the induction of lipase expression by various inducer molecules is not understood. Lipase expression in fungi, like *Penicillium simplicissimum* (52), *Geotrichum candidum* (47), *Aspergillus japonicus* (56), *Beauveria bassiana* GK 2116 (19), and *Oospora fragrans* (46), can be induced with oils and fatty acids. In other species, such as *Sulfolobus shibatae* (20) and *Malassezia furfur* (41), lipase expression can be stimulated by various types of detergents, such as Tween (poly-oxyethylene-sorbitan). For some *Pseudomonas* species (55), fatty acid alcohols are mentioned as the inductive component. For *Acinetobacter calcoaceticus* BD413, aliphatic alkane *n*-hexadecane (23) has been identified as an inducer. In recent years, lipase expression of *Pseudomonas alcaligenes* M-1 has been studied extensively (13), and a fed-batch fermentation process was developed. It was found that lipase synthesis can be switched on by a limited number of natural oils, such as soybean oil, and some unsaturated fatty acids, such as oleic acid. A significant synthesis of lipase was observed only for the combination of a batch phase based on a minimal citrate medium and a feed phase based on soybean oil. Expression of lipase was observed only after the start of the feed with soybean oil (13).

Numerous bacterial lipase genes have been identified and sequenced in the past (15, 21). Nevertheless, the lipase expression transcriptional regulation mechanism in particular is poorly understood. Almost no reports about the transcriptional regulation of lipase genes from pseudomonads are available. It is known that lipases from a number of *Pseudomonas* and *Burkholderia* species are expressed from a unique type of operon, where the structural gene for lipase (*lipA*) is followed by a gene coding for a helper protein (*lipB*). In the past, this lipase helper protein—LipB—has been investigated for its suspected role in the regulation of lipase expression (12). It is now generally accepted that the lipase helper protein plays a role in periplasmic lipase folding and not in transcriptional regulation (22). For *Acinetobacter calcoaceticus* BD413 (23) and *Rhizobium etli* (53), the presence of a σ70 (RpoA-dependent) promoter as the lipase transcriptional regulon has been reported. For *P. alcaligenes* M-1, the transcription start of the lipase operon was mapped (7) downstream of a typical σ54-type promoter with the characteristic GG-N10-GC sequence at transcription start positions −12 to −24 (9, 30). Also, for *Pseudomonas aeruginosa* (22) and *Pseudomonas* species M-12-33 (36), a σ54 (RpoN-dependent) promoter in front of the lipase operon has been postulated based on sequence data. These promoters operate in conjunction with an upstream activator sequence (UAS) essential for the binding of a regulatory protein. Upon the binding of the regulatory protein to

* Corresponding author. Mailing address: Pharmaceutical Biology, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. Phone: 31503632558. Fax: 31503633000. E-mail: w.j.quax@rug.nl.

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TABLE 1. Bacterial strains

Strain	Description[a]	Reference or source
Pseudomonas alcaligenes strains		
Ps93	Restriction negative, modification positive	14
Ps537	High-lipase-producing isolate of strain M-1	7
Ps770	Neo^r [pJRDlipAB]	This study
Ps824	*lipA* mutant	This study
Ps1018	Ps770 with cosmid 201; Neo^r Tet^r	This study
Ps1029	Ps770 with pLAFR3; Neo^r Tet^r	This study
Ps1030	Ps770 with cosmid 726; Neo^r Tet^r	This study
Ps1034	Ps770 with cosmid 71; Neo^r Tet^r	This study
Ps1036	Ps770 with cosmid 505; Neo^r Tet^r	This study
Ps1039	Ps537 with cosmid 505; Tet^r	This study
Ps1040	Ps537 with cosmid 71; Tet^r	This study
Ps1041	Ps537 with cosmid 726; Tet^r	This study
Ps1042	Ps537 with cosmid 201; Tet^r	This study
Ps1049	Ps537 with cosmid 201E-H; Tet^r	This study
Ps1100	Restriction negative, modification positive; *lipR* mutant; Tet^r	This study
Escherichia coli strains		
Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*77679 galU galK rpsL*::Str^r *endA1 nupG*	17
K802	*hsdR2 galK2 galT22 mcrA mcrB1 metB1 supE44*	42
DH10B	F$^-$ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	Invitrogen

[a] pLAFR3 (strain Ps1029) is described in reference 49. Str^r, streptomycin resistance.

this UAS and to σ54 RNA polymerase, the expression of the gene is induced. Within the upstream region of the lipase promoter region of *P. alcaligenes*, a sequence that is homologous to the Nif type of UASs (consensus, TGT-N10-ACA [3, 25]) was identified (7). Mutational analysis of this UAS confirmed an involvement in lipase regulation (7).

One rational approach for finding regulatory proteins is to search for negative phenotypes followed by complementation using libraries of *P. alcaligenes* genomic DNA. However, mutagenesis of *P. alcaligenes* M-1 with physical and chemical agents yielded lipase-negative phenotypes at high frequency, resulting in too many mutants to work with (data not shown). Consequently, in order to identify factors essential in the regulation of the expression of lipase from *P. alcaligenes*, we have developed a selection for positive phenotypes—the phenotype enhancement method. A cosmid library of random chromosomal fragments from *P. alcaligenes* was introduced into a high-producing lipase strain and subsequently a sensitive screening for hyperproducing strains was done under various growth conditions. One of the elements found, a two-component regulatory system, is a subject of this study.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and chemicals. For this study, bacterial strains from the species *Pseudomonas alcaligenes* and *Escherichia coli* were used. The introduced genetic modifications and plasmids used are shown in Table 1.

All bacterial strains listed in Table 1 were propagated in 2× TY (16 g/liter Bacto tryptone, 10 g/liter Bacto yeast extract, 5 g/liter NaCl, pH 7.0) as a liquid or solid medium, unless otherwise stated. Two screening tests containing minimal salt medium (for composition, see reference 14) were supplemented with either 0.2% soybean oil (test I) or 0.5% sodium lactate (test II). Screening test III consisted of brain heart infusion (BHI) medium (37 g/liter BHI; Difco) supplemented with 17 mM *n*-hexadecane.

Bacterial strains containing plasmids or cosmids were grown either in medium or on agar plates with tetracycline at 5 mg/liter for *P. alcaligenes*, tetracycline at 25 mg/liter for *E. coli*, ampicillin at 100 mg/liter for *E. coli*, and neomycin at 10 mg/liter for *P. alcaligenes*.

Restriction enzymes, T4 DNA ligase, *Pwo* polymerase, and *Taq* polymerase were obtained from Gibco-BRL or Fermentas and used as recommended by the manufacturer. Soybean oil was purchased from Cargill (Belgium), sodium lactate was from Merck (50% pure), and *n*-hexadecane was kindly provided by K. Hellingwerf (University of Amsterdam).

Construction of plasmids, strains, and DNA probes. (i) Construction of cosmid library. A cosmid library was constructed by the insertion of partially digested Sau3A chromosomal fragments (ranging from 20 to 30 kb in length) of the *P. alcaligenes* wild-type strain, M-1, into the BamHI-opened cosmid pLAFR3 (49).

(ii) Construction of a *lipR* mutant strain. In order to create a *lipR* mutant by insertional inactivation, an 807-bp fragment of an internal *lipR* part (bp 296 to 1116 relative to the translation start site) was amplified using two primers: forward (5′ CCAAGCCCTTCGATCGCGACGAGAATGCT 3′, where the PvuI site is underlined and the base shown in boldface was introduced to generate a frameshift mutation) and backward (5′ GCGCTCGATGGAATTCTCCAGCT CGCGCA 3′, where the EcoRI site is underlined). A purified and PvuI-EcoRI-digested internal *lipR* fragment of 807 bp was introduced into a pBR322 vector of 3,737 bp (PvuI-EcoRI generated) followed by *E. coli* Top10 transformation. Tetracycline-resistant (Tet^r) transformants were selected on LB agar plates containing 25 mg/liter tetracycline, and plasmid DNA was isolated, sequenced, and electroporated to *P. alcaligenes* strain Ps93. Integrants were selected on 2× TY plates containing 5 mg/liter tetracycline.

(iii) Construction of *lipR*-expressing plasmid. To construct the pME6032LipR plasmid, a *lipR* gene (1,416 bp) was amplified from cosmid 505 with the forward (5′ CGAGAGGAATTCATGCCGCATATCCTCATCGT 3′) and backward (5′ TGTCACAGATCTTCAGGAGCCGGTCGCCG 3′) primers carrying, respectively, EcoRI and BglII restriction sites (underlined). After digestion, this product was ligated into EcoRI-BglII-cut pME6032 shuttle vector (18), resulting in pME6032LipR.

(iv) Promoter DNA fragments. Probes P_{lipA} (lipase promoter sequence) were obtained by PCR with *Taq* polymerase (Fermentas) from plasmid pJRDlipAB (13) by use of the primers ForLipA (5′ CCCCTGGCTGGCAGGCGGCAG 3′) and BackLipA1 (5′ TTGGTCTTGGTGTAGCCGGT 3′) to generate the 367-bp probe P_{lipA}367 and BackLipA2 (5′ GCCGGTACTTCAGCAGGCT 3′) to generate the 199-bp probe P_{lipA}199.

Isolation of cosmid DNA from *E. coli* was performed with the cetyltrimethylammonium bromide method (35). Small-scale purification of cosmid DNA from *P. alcaligenes* was done with the QIAprep spin plasmid kit and large-scale iso-

lation with the QIAfilter plasmid midi kit, both according to Qiagen's instructions. Plasmids and cosmids were transformed to *E. coli* as described by Calvin and Hanawalt (5). For *P. alcaligenes* transformation, the method of Wirth et al. (59) was applied, with the modification that all treatments were performed at room temperature. Purification of PCR products was done using the PCR purification kit from Qiagen according to the supplier's instructions. For Southern hybridizations (48), detection of DNA-containing filters was executed with DNA probes labeled with the ECL labeling kit from Gibco-BRL (following the instructions of the supplier).

RNA isolation, cDNA preparation, and qRT-PCR. Total RNA was isolated with a High Pure RNA isolation kit (Roche) from the *P. alcaligenes* cells collected from minimal tributyrin plates. Total RNA was quantified with an ND-1000 spectrophotometer (NanoDrop). Reverse transcription was conducted in a total volume of 20 μl. Random nanomers (2.5 μl of 1.65 μg/μl; Isogen) were mixed with 2.5 μg isolated RNA and 2 μl 10 mM deoxynucleoside triphosphate mix, incubated for 5 min at 65°C, and cooled on ice. Subsequently, 4 μl 5×-concentrated first-strand buffer (Invitrogen), 1 μl 100 mM dithiothreitol, 1 μl RNaseOUT (40 U/μl; Invitrogen), and 1 μl SuperScriptIII reverse transcriptase (200 U/μl; Invitrogen) were added, and the total volume was adjusted with diethyl pyrocarbonate-treated water to 20 μl and kept at 25°C for 5 min. A reverse transcription reaction was performed at 55°C for 60 min and was stopped by heating at 70°C for 15 min. Synthesized cDNA was further used in quantitative real-time PCR (qRT-PCR) that was carried out in 96-well microtiter plates in a final reaction volume of 20 μl by use of an iCycler iQ real-time PCR instrument (Bio-Rad). Each reaction mixture consisted of 1 μl cDNA, 10 μl iQ SYBR green supermix (Bio-Rad), 1 μl forward primer (10 μM), 1 μl backward primer (10 μM), and 7 μl water. Reaction mixtures were initiated with a 4-min incubation at 95°C followed by up to 40 cycles at 95°C for 10 s (denaturation) and 60°C for 45 s (primer annealing/extension). The increase in fluorescence was measured automatically during PCR. Oligonucleotide pair primers for the *lipA* gene and the 16S rRNA housekeeping gene were designed with PerlPrimer software. Cycle threshold values were determined using the software provided with the thermal cycler. All cDNA samples were amplified in triplicate and normalized against a triplicate of the housekeeping gene 16S rRNA in the same plate.

Protein expression, preparation, and purification. Erlenmeyer flasks containing 2× TY medium (supplemented with tetracycline at 5 mg/liter or 25 mg/liter, if necessary) were inoculated (100× dilution) with overnight cultures of Ps537, Ps1039, Ps1100, *E. coli* DH10B, and *E. coli* DH10B/pME6032LipR, respectively. Cultures were grown at 30°C for 20 h for *Pseudomonas* strains and for 8 h for *E. coli* strains. Cell cultures were harvested by centrifugation. The pellets were resuspended in 50 mM Tris, pH 8.0, 2 mM EDTA and sonicated (output 4, 40% duty cycle on a Sonifier 250; Branson), and the membrane fractions were removed by centrifugation (30 min, 4°C, and 17,000 × *g*). *E. coli* cell-free protein extracts were subjected to affinity chromatography on a heparin-Sepharose column and eluted with a NaCl gradient, using the Ákta Explorer system (GE Healthcare). The concentration of proteins was determined by the Bradford method (1) using bovine serum albumin as a protein standard (Pierce). The protein samples were analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel stained with Coomassie brilliant blue (26). *Pseudomonas* cell-free protein extracts and *E. coli* partially purified protein fractions were used further in electrophoretic mobility shift assays (EMSA). To identify overexpressed in *E. coli* LipR protein, the protein band of interest was extracted from the gel, trypsin digested, and analyzed with matrix-assisted laser desorption ionization–time of flight mass spectrometry. Protein identification based on mass spectra was done by peptide mass fingerprinting using Mascot software (Matrix Science).

Screening tests for lipase hyperproduction strains. The *P. alcaligenes* strain Ps770 is an overproducer of lipase and contains multiple copies of plasmid pJRDlipAB carrying *lipA* and *lipB* (13). This strain was used for transformation by electroporation of the cosmid library. The selection of the lipase-hyperproducing *P. alcaligenes* transformants from the collection of cosmid-containing strains was performed using a high-throughput screening based on microtiter plate assays. Since *P. alcaligenes* M-1 is not able to grow on glucose as a single carbon source (13), other carbon sources were used as described in Materials and Methods. The media for screening tests I, II, and III were used to grow cosmid strains in a 96-well microtiter plate in a volume of 100 μl. Cells were grown at 35°C for 24 h (test III) or 48 h (tests I and II) in a CO_2 incubator filled with 5% CO_2. From each suspension sample, an aliquot of 10 μl was transferred automatically (Tecan Robotics) into a well on an agar plate supplemented with 2% (vol/vol) tributyrin oil. The incubation of these tributyrin oil plates was done at 37°C for 48 h. The area of clarification (halo) was automatically recorded using image analysis equipment. Also, the A_{600} values from the cultures grown in microtiter plates were directly measured with a reader (Anthos HT-III). The lipase activity was calculated by dividing the value of the area of clarification by the A_{600} value.

Fermentation. The fed-batch fermentation process with a feed of soybean oil was performed in 10-liter fermentors according to the standard procedure as mentioned in reference 13. Lipase activity was measured as described in the same publication. Data points were fitted to a trend line according to the least-mean-squares method.

Gel retardation. A gel retardation assay, with cell-free protein extracts (from the Ps537, Ps1039, and Ps1100 strains) or purified on heparin-Sepharose column proteins (from *E. coli* DH10B and *E. coli* DH10B/pME6032LipR strains), was carried out as described by Ebbole and Zalkin (10). The DNA probes $P_{lipA}367$ and $P_{lipA}199$ were radioactively end labeled with the T4 polynucleotide kinase by use of [γ-^{32}P]ATP. The protein fractions and a probe were premixed on ice in a binding buffer [20 mM Tris-HCl at pH 8, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 10 mg/liter poly(dI-dC), and 0.1 g/liter bovine serum albumin]. After 20 min at 37°C, samples were loaded on a nondenaturing gel (5% Tris-borate-EDTA [TBE] gel; Bio-Rad). Gels were run at 100 V in 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2.8 mM EDTA; solution at pH 8.3), dried, and autoradiographed.

Sequence analysis. Sequencing was carried out on an Applied Biosystems 373A DNA sequencer by use of an ABI Prism dye termination cycle sequencing ready reaction kit with AmpliTaq DNA polymerase.

Nucleotide sequence accession numbers. The sequences from this study have been deposited in the NCBI database under GenBank accession numbers EU432185 (LipR) and EU432186 (LipQ).

RESULTS

A selection system for lipase regulatory genes. A selection of regulatory genes is usually performed via transposon mutagenesis or similar knockout techniques. In the case of *Pseudomonas alcaligenes*, this led to hundreds of mutants impaired in lipase production. In order to obtain a direct selection for factors limiting lipase expression, we set up a positive selection system based on phenotype enhancement. This positive selection system is based on the introduction of a cosmid library into a lipase-overproducing strain, Ps770, which harbors a multicopy plasmid (neomycin-resistant [Neo^r] pJRDlipAB [13]) carrying the *lipA* and *lipB* genes, followed by quantitative lipase assays.

In *E. coli* K802, 754 transformants (Tet^r) were collected with cosmids containing random fragments of *P. alcaligenes* chromosomal DNA. Subsequently, 531 independent cosmids could be transferred to *P. alcaligenes* strain Ps93 (restriction negative, modification positive), giving a cosmid library in *P. alcaligenes*. Cosmid DNA was isolated from these 531 Ps93 strains, and all cosmids were transformed individually to strain Ps770 by electroporation. In total, 485 cosmids could be successfully introduced into the lipase-overproducing strain Ps770, resulting in colonies that are both Neo^r and Tet^r. A screen involving three different microtiter tests was developed, and all 485 cosmid-containing strains were tested in duplicate and judged on their lipase activity.

The initial screening led to the selection of 42 strains with a significantly high lipase expression in one of the three tests compared to the Ps1029 strain containing the empty pLAFR3 vector (49). The examination of these 42 strains was repeated in quadruplicate. Out of them, 20 strains were found to score considerably higher than strain Ps1029 in the soybean oil test. The 20 cosmid strains were ranked by their lipase levels (clarification zone on tributyrin oil agar plate/optical density at 600 nm) obtained in 10×-diluted minimal salt medium supplemented with soybean oil. Table2 presents the results for four cosmids (505, 71, 201, and 726). DNA from all 20 lipase-

TABLE 2. Microtiter test results

Cosmid	Lipase productivity of strain with indicated cosmid in[a]:		
	Medium 380 + soybean oil	Medium 380 + lactate	BHI + hexadecane
505	63.5 ± 10.4	28.8 ± 3.1	15.0 ± 3.6
71	40.3 ± 11.4	27.3 ± 5.3	16.7 ± 2.5
201	39.0 ± 5.6	18.0 ± 4.2	10.0 ± 1.4
726	36.8 ± 4.5	25.3 ± 4.9	21.0 ± 8.7
pLAFR3	20.8 ± 2.1	11.5 ± 1.8	11.5 ± 2.9

[a] Lipase productivity is the size of the clarification zone (mm^2) on tributyrin oil agar plate/optical density at 600 nm for the 4 selected cosmid strains from the 20 lipase-stimulating strains chosen after microtiter assay done in quadruplicate.

stimulating cosmids was isolated and transformed via electroporation to *P. alcaligenes* strain Ps537, which carries only a single copy of the lipase operon in the chromosome. Four of the resulting strains, Ps1039, Ps1040, Ps1041, and Ps1042 (containing cosmids 505, 71, 726, and 201, respectively), gave a clarification zone (halo) much larger than that of the wild-type strain on a tributyrin oil plate (Fig. 1). This indicates that the four cosmids harbor either a factor that stimulates the lipase expression or an esterase or lipase gene that hydrolyzes tributyrin oil. This latter possibility was ruled out by testing the four cosmids in a lipase-negative strain, Ps824, which carries a deletion of the lipase gene. None of the four cosmids showed hydrolysis of tributyrin oil after transfer to strain Ps824 (data not shown).

Characterization and analysis of four lipase-stimulating cosmids. The four lipase-stimulating cosmids were found to share an overlapping fragment of 5.6 kb, as could be deduced from their restriction enzyme patterns. Maps of the cosmids are depicted in Fig. 2. From cosmids 201 and 505, parts of this 5.6-kb fragment were cloned separately into pLAFR3 (49) and transformed into the Ps537 strain by electroporation. A fragment of 4.5 kb from cosmid 201 in pLAFR3 (49) still stimulated lipase expression in *P. alcaligenes* (Fig. 2). This strain, Ps1049, shows the same size of lipase-clearing zone as for the four stimulating cosmids in Ps537 (Fig. 1). The 4.5-kb EcoRI-HindIII fragment of cosmid 201 (Fig. 2) was cloned into pUC18, transformed to *E. coli* Top10 cells, and sequenced. Sequence analysis of the lipase-stimulating fragment revealed an insert of 4,377 bp including an N-terminally truncated open reading frame (ORF) with homology to NtrB-like proteins (sensor kinases), a second complete ORF for an NtrC-like homologue (response regulators), and a third ORF missing the C-terminal sequence. The lacking sequence of the first ORF was obtained after sequencing a part of insert from cosmid 505. Finally, after assembly of the sequences, the first ORF gave a gene encoding a protein (984 amino acids) which shows significant identity (79%) with the CbrA protein from *P. aeruginosa* (37) and a somewhat lower degree of identity with other phosphorylating NtrB proteins (38) (members of the NtrBC family of two-component regulatory proteins). This protein was named LipQ. In Table 3, the identity scores of LipQ and LipR with the most related proteins are shown. The second ORF (encoding a protein of 471 amino acids) shows homology (about 35% identity) with DNA binding NtrC-like proteins (38) and a remarkable identity of 87% with the *P. aeruginosa* CbrB protein (37). This ORF was named *lipR*. There is an intergenic region of 65 bp between the *lipQ* and *lipR* genes.

The incomplete ORF protein, called OrfZ, has a high level of homology with polyadenyl polymerases. It shares a significant match of 81% at the peptide level with poly(A) polymerase of *Pseudomonas syringae* (4) and 81% with poly(A) polymerase of *P. aeruginosa* (51) and a 79% identity with *P. fluorescens* poly(A) polymerase (40). Moreover, the incomplete OrfZ has 58% identity with poly(A) polymerase of *Shigella boydii* (61) and 58% with poly(A) polymerase of *Escherichia coli* (45, 58). The fact that the ORF—*orfZ*—homologous to the *pap* genes of different microorganisms is incomplete on the smallest lipase-stimulating fragment implies that it has no effect on lipase stimulation, and there is no reason to believe that this downstream gene is involved in lipase regulation.

Comparison of LipQR with other two-component regulatory systems. Sequence alignment of the *lipQR* operon with two-component regulatory systems reveals a notable similarity of LipQR with NtrBC-like two-component regulatory proteins. The LipR protein shows the most resemblance with the NtrC type of DNA binding proteins of other gram-negative bacteria (Fig. 3A), whereas LipQ has a significant homology to the NtrB type of histidine kinases (Fig. 3B). The protein sequences were aligned using the Megalign algorithm of Lasergene software, and the conserved residues are shaded. The relevant elements of regulatory proteins, like the AAA domain (ATPase-associated activity domain) and the σ54-interacting domain, are marked in Fig. 3B (R1 to R7), while the N-terminal REC domain (with a phosphoacceptor site that is phosphorylated by kinase homologue) and the C-terminal DNA binding helix-turn-helix domain were not included in the figure. In LipR, the aspartic acid at position 52 represents a strictly conserved residue that lines up with the functionally important phosphorylation site in the NtrC proteins (34). Furthermore, the seven well-conserved regions of NtrC proteins can also be recognized in LipR. These seven regions correspond precisely with the seven regions in the central part of NtrC proteins that have been identified as the σ54 factor interaction domains (34). Especially, the sections that fit the consensus for ATP-binding regions (R1, **G**E-G-G**K**E; R4, G**G**T-FL**DE**IG; R6, V**R**-I-AT—L [boldface indicates residues strictly conserved among all ATP binding sequences and hyphens indicate positions that show more variability] [23a]) are strongly conserved between LipR and the NtrC proteins (Fig. 3A). In contrast, in the hydrophobic R2 region, LipR and CbrB have like other

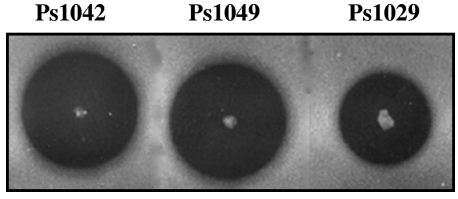

FIG. 1. Lipase-enhancing cosmids were selected on the basis of the halo size around a colony on a plate with tributyrin oil (1% [vol/vol]). The halo size is a measure of the amount of lipase activity made by this colony. Strains: Ps1042, with cosmid 201 in Ps537; Ps1049, carrying the subcloned 4.5-kb EcoRI-HindIII fragment of cosmid 201 in pLAFR3; and Ps1029, with pLAFR3 in Ps537.

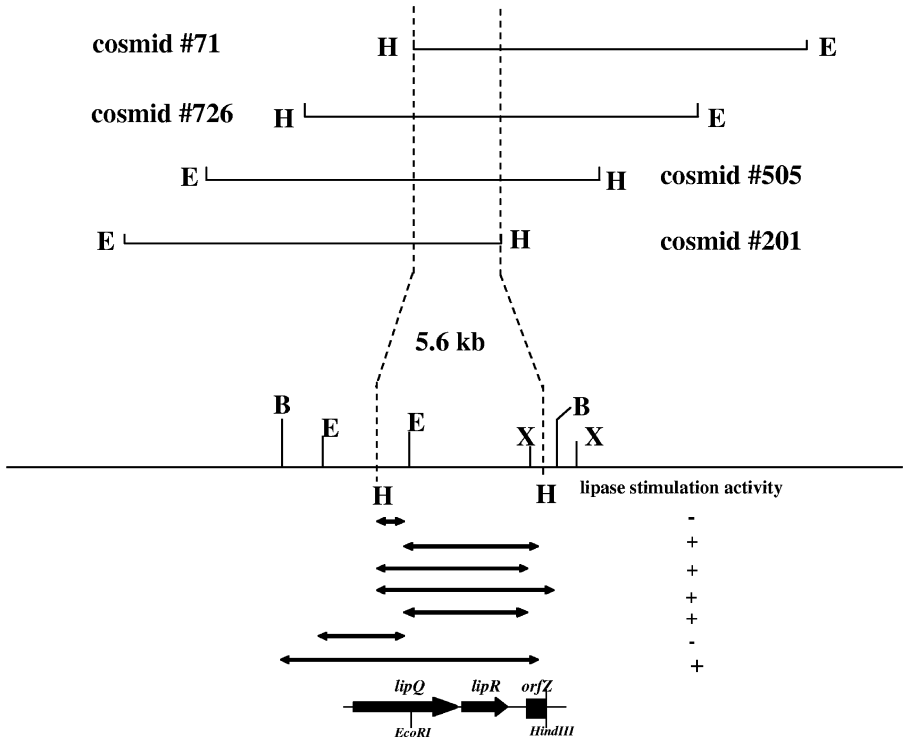

FIG. 2. The restriction enzyme patterns of the four lipase-stimulating cosmids (71, 201, 505, and 726) are illustrated as four lines showing their overlapping region of 5.6 kb. Beneath these lines, the fragments from cosmid 201 and cosmid 505 subcloned in pLAFR3 are exhibited, and their ability (+) or inability (−) to stimulate lipase activity on a tributyrin oil plate is cited. At the bottom, the 4.5-kb EcoRI-HindIII segment of cosmid 201 showing the location of the three ORFs on this lipase-stimulating fragment is presented.

TABLE 3. Comparison of a selected group of proteins with LipR and LipQ from several protein databases

Protein name[a]	% Match (no. of matching amino acids)[b]	Protein length (amino acids)	Accession no.[c]
Proteins with LipR			
NtrCAzobr	38 (197)	481	P45671
NtrCKlepn	40 (199)	469	P03029
NtrCBrasr	35 (176)	480	P10576
NtrCRhime	36 (185)	484	P10577
NtrCEcoli	39 (195)	469	P06713
NtrCSalty	39 (193)	469	P41789
NtrCProvu	37 (188)	473	P28787
NifAAzobr	28 (178)	625	P30667
NifAKlepn	33 (185)	524	P03027
NifABraja	31 (186)	582	P05407
CbrBPAO	87 (417)	478	BAB20867
MhaRPspu	84 (407)	480	AAY16576
Proteins with LipQ			
NtrBRhoca	12 (130)	356	P09431
NtrBBrasr	13 (134)	377	P10578
NtrBKlepn	12 (123)	349	P06218
NtrBSalty	12 (122)	349	P41788
NtrBEcoli	11 (118)	349	P06712
NtrBAzobr	12 (125)	400	P45670
NtrBProvu	11 (117)	348	P28788
KinABacsu	18 (184)	606	P22863
KinBBacsu	14 (142)	429	Q08430
KinCBacsu	11 (121)	428	P39764
CbrAPAO	79 (786)	983	BAB20866
MhaSPspu	81 (804)	991	AAY16575

[a] Name of protein as abbreviated in database.
[b] % Identity with LipR or LipQ on the amino acid level. The numbers of amino acids that match LipR or LipQ are shown in parentheses.
[c] Accession number of abbreviated protein in protein database.

regulatory proteins a conserved cysteine, whereas NtrC proteins have a methionine (Fig. 3A). The well-conserved R3 region consisting of two α-helices separated by a turn (34) is clearly identifiable in LipR, and this is one of most important characteristic regions for σ54 regulatory proteins. The other regions (R5 and R7 [Fig. 3B]) in LipR are fairly homologous to NtrC-like proteins. LipR has a helix-turn-helix motif in the C-terminal part of the protein, like all other NtrC-related proteins. Chou-Fasman (6) secondary structure prediction gives the turn at exactly the same position as for the NtrC proteins, but the lengths of the two α-helices differ from those for NtrC proteins. The predicted first α-helix of the helix-turn-helix from LipR is longer than for NtrC proteins. Interestingly, the CbrB protein from *P. aeruginosa* (478 residues; accession no. BAB20867) shows 87% identity to LipR (471 residues). The seven well-conserved internal regions and the C-terminal helix-turn-helix are present in CbrB as well. The proteins LipR and CbrB have a high variability only in two regions, where CbrB also has the following additional amino acids compared to LipR: residues 114 to 144 and residues 397 to 414.

Furthermore, Fig. 3A presents a partial alignment of LipQ protein with other NtrC homologues, including their highly conserved histidine kinase A domain (dimerization and phosphoacceptor domain) region; the phosphorylated histidine characteristic for this type of kinase is marked. The N terminus of LipQ up to approximately 450 amino acids corresponds to transmembrane stretches that are followed by a PAS domain (62) (signaling sensor domain) that goes from 632 to 700 in the amino acid sequence. When comparing CbrA (accession no.

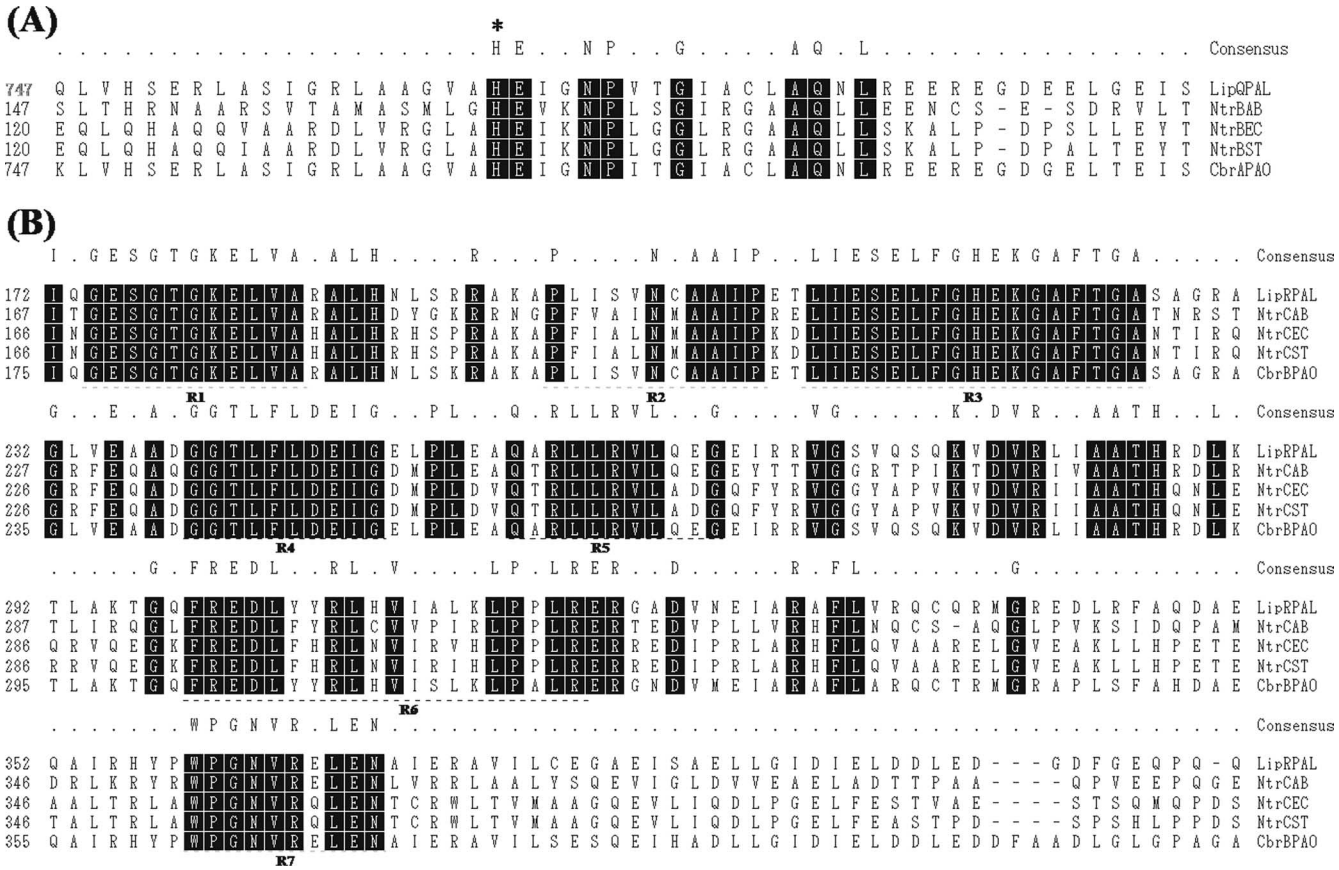

FIG. 3. Sequence alignment of the deduced amino acid sequences of LipQ and LipR with different homologues of two-component systems. The Clustal method with percent accepted mutation residue weight table (Lasergene technology) has been used for the alignment. Dark-shaded residues represent the amino acids that are identical in all aligned proteins. (A) The partial alignment of LipQ from *P. alcaligenes* M1 (LipQPAL [this study]) with NtrB proteins of *Azospirillum brasilense* (NtrBAB [28]), *Salmonella enterica* serovar Typhimurium (NtrBST [25]), *Escherichia coli* (NtrBEC [31]), and *Pseudomonas aeruginosa* (CbrAPAO [37]) is displayed. The presented part of the sequences refers to the highly conserved His kinase A domain, including the position of the autophosphorylation site, which is indicated with an asterisk. (B) The alignment of LipR from *P. alcaligenes* M1 (LipRPAL [this study]) with NtrC proteins of *Azospirillum brasilense* (NtrCAB [28]), *Escherichia coli* (NtrCEC [31]), *Salmonella enterica* serovar typhimurium (NtrCST [25]), and *Pseudomonas aeruginosa* (CbrAPAO [37]) is demonstrated. The conserved regions (R1 to R7) that contain the most common features of two-component regulators as discussed in the text are displayed below the alignments. The leftmost numbers correspond to amino acid numbers.

BAB20866) with LipQ, the similarity is evident from the lengths (CbrA, 983 residues; LipQ, 984 residues) and the high amino acid identity (79%). In contrast to other NtrB homologues in our alignment, CbrA and LipQ display strikingly long N-terminal sequences (about 600 amino acids longer) corresponding to transmembrane domains. At the beginning of the transmitter domain at position 766 in LipQ (position 766 in CbrA), a conserved histidine can be found within a conserved region (Fig. 3A). This histidine is extremely conserved in prokaryotic sensor transduction histidine kinases, and it represents the autophosphorylation site. The overall identity of the transmitter domain of LipQ in comparison with that of NtrB proteins is moderate; however, the similarity of functionally important residues and regions is apparent (Fig. 3A).

Overexpression of *lipQR* enhances lipase expression in *P. alcaligenes* during fermentation. An additional observation supporting the conclusion that LipQR acts as the regulator for lipase expression in *P. alcaligenes* comes from fermentation studies. The overexpression of *lipQR* (in strains Ps1039, Ps1040, Ps1041, and Ps1042) results in a large halo on a tributyrin oil plate compared to what is seen for strain Ps537 with empty vector pLAFR3 (49). As an example, the halo formation of strain Ps1042 is shown in Fig. 1.

In a mimicked large-scale fermentation (at a 10-liter scale), strain Ps1039 containing cosmid 505 with LipQR was compared with host strain Ps537 containing the empty vector. A fed-batch fermentation was performed according to the protocol described previously with a batch medium based on citric acid and a feed phase based on soybean oil (13). The lipase yield data plotted against the fermentation time are shown in Fig. 4. The best fit of the data was found with a polynomial (third-order) trend line. The trend line was calculated within an R_2 of 0.9858 for the Ps537 curve and an R_2 of 0.9934 for the Ps1039 curve. As can be seen from Fig. 4, the lipase production by Ps1039 not only starts at a higher rate but also extends to a level that is threefold higher than that seen for the control. It was verified that during fermentation no loss of the cosmid vector occurred. The other LipQR-overproducing strains

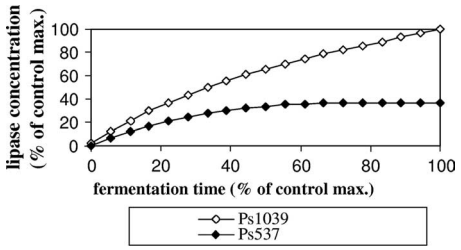

FIG. 4. Fermentation results of *P. alcaligenes* strains Ps537 and Ps1039 (Ps537 containing cosmid 505) are demonstrated. The *x* axis represents the fermentation time as a percentage of the maximal (max.) fermentation time and the *y* axis the lipase activity in as a percentage of the maximal lipase activity.

(Ps1040, Ps1041, Ps1042, and Ps1049) also showed a threefold lipase increment compared to the control (data not shown).

As described before, in the Ps1036 strain both cosmid 505 and expression plasmid pJRDlipAB (13) are present. However, during the fermentation of this strain in a 10-liter fermentor, retarded growth and severe loss of the cosmid 505 and/or the plasmid pJRDlipAB (13) was observed, rendering conclusions on yield impossible.

Inactivation of the *lipR* gene down-regulates lipase expression. In order to investigate the effects of chromosomal *lipR* inactivation, we constructed a *lipR*-negative strain by insertional inactivation, and amplification with *Pwo* polymerase was conducted on an internal part of *lipR* by use of forward and backward primers as described in Materials and Methods. Tetracycline (5 mg/liter)-resistant colonies were checked for the presence of the integration unit by use of Southern hybridization with the PCR fragment as a probe (data not shown). One selected strain was used in further experiments; it was named Ps1100. The *lipR*-inactivated strain, Ps1100, was tested for

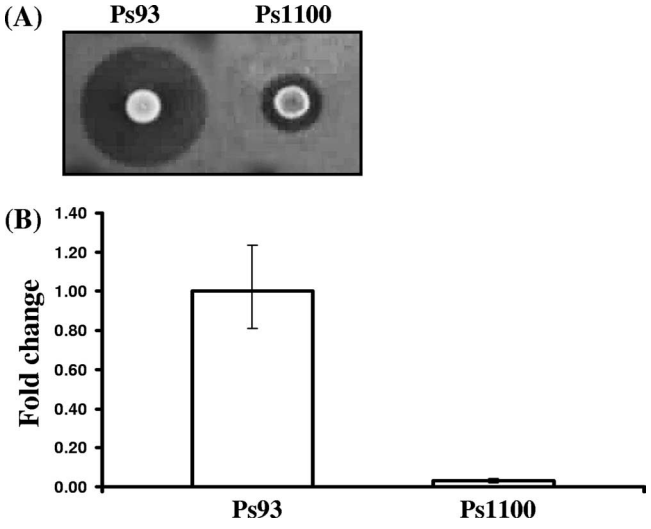

FIG. 5. Effects of *lipR* inactivation on lipase expression. (A) Lipase activity on a tributyrin oil plate for the Ps93 strain (restriction-negative wild type) and the Ps1100 strain (Ps93 with inactivated *lipR*). (B) Relative quantity chart for lipase gene expression. The Ps93 strain sample (calibrator) is given an arbitrary quantity of 1; the Ps1100 strain sample presents the quantity relative to the calibrator. Cells were harvested from tributyrin oil plates after incubation at 37°C for 48 h, as shown in panel A.

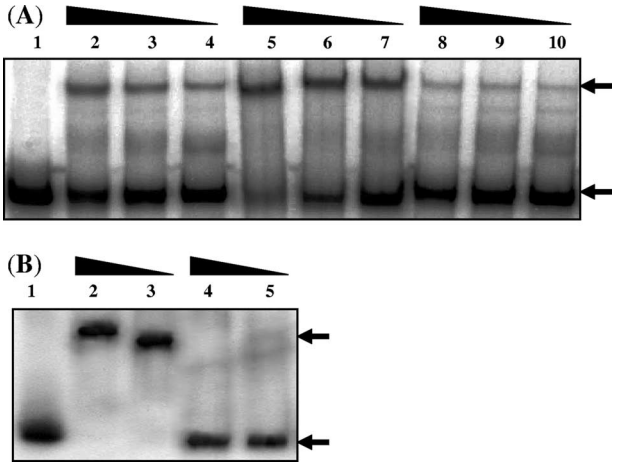

FIG. 6. Gel retardations. Nucleotide DNA fragments (P_{lipA}367 and P_{lipA}199) amplified by PCR, corresponding to the lipase promoter and its UAS, were radioactively end labeled. They were then incubated with decreasing amounts of protein samples. Finally, the mixtures were analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel (5% TBE gel; Bio-Rad). Top and bottom arrows indicate retarded and nonretarded bands, respectively. (A) P_{lipA}367 probe with/without cell-free protein extracts from *Pseudomonas* strains at concentrations ranging from 10 μg to 2.5 μg. Lanes: 1, no protein extract added; 2 to 4, probe samples incubated with proteins from the Ps537 strain; 5 to 7, with proteins from Ps1039 strain; 8 to 10, with proteins from Ps1100 strain. (B) P_{lipA}199 probe with/without purified protein samples from *E. coli* strains at concentrations of 10 μg and 5 μg. Lanes: 1, no protein added; 2 and 3, purified proteins from the DH10B/pME6032LipR strain; 4 and 5, with purified proteins from the DH10B strain.

lipase expression on a tributyrin oil plate and found to give a halo size reduced from that seen for the Ps93 strain (Fig. 5A). The reduced clearing zone present for the Ps1100 strain could be the result of low basal levels of lipase transcription in the absence of LipR and/or the result of resident esterase activity. Further, to verify the expression levels of the *lipA* gene for two *P. alcaligenes* strains, we collected bacterial cells from the agar plates supplemented with tributyrin oil. Figure 5A represents the plate with colonies collected for RNA isolation. Gene expression, quantified by the qRT-PCR method and the comparative critical threshold ($\Delta\Delta C_T$) method, revealed that inactivation of the *lipR* gene (Ps1100 strain) significantly decreased lipase mRNA levels (Fig. 5B). The relative expression level of the *lipA* gene was normalized to 16S rRNA, and the level of transcripts was determined relative to that of the Ps93 strain. The Ps1100 mutant gave a relative lipase expression level of 0.03 relative to an assigned arbitrary quantity of 1 for the Ps93 strain (Fig. 5B). These data support our findings that lipase transcript is down-regulated in the absence of LipR protein.

DNA binding activity in crude protein extracts from *P. alcaligenes*. To test the binding properties of cell extracts from different *Pseudomonas alcaligenes* strains (Ps537, Ps1039, and Ps1100) to the lipase promoter region P_{lipA}367, a 367-nucleotide fragment corresponding to the lipase promoter regulatory region from −183 to +184 was amplified by PCR. The DNA probe was used in gel retardation assays with decreasing amounts of protein cell extracts. DNA band shifts indicating a protein-DNA complex formation were detected, as shown in Fig. 6. Figure 6A, lanes 1 to 4, presents the mobility of the

lipase promoter region $P_{lipA}367$ upon incubation with 0 μg, 10 μg, 5 μg, and 2.5 μg of Ps537 crude protein extract, respectively. The amount of shifted fragment decreased proportionally with a decreasing amount of the crude protein extract that was added to the assay mixture. A similar observation was made when crude protein extract from the Ps1039 *lipR* overexpression strain (with cosmid 505) was used in gel retardation (Fig. 6A, lanes 5, 6, and 7). In all cases, the retarded complex migrated to the same position. However, the amount of the DNA probe shifted upon incubation with cell extract of LipR-overproducing strain Ps1039 was higher than for Ps537 cell extract.

Surprisingly, the gel shift assay with a cell-free protein extract from the Ps1100 strain still shows some binding to lipase promoter sequence $P_{lipA}367$. Lanes 8 to 10 of Fig. 6A present the mobility of the lipase promoter after incubation with 10 μg, 5 μg, and 2.5 μg of Ps1100 crude protein extract, respectively. As can be observed by the presence of retarded bands, the binding increased proportionally with increasing concentrations of proteins from cell extract. Overall, however, the intensity of the retarded band is low compared with what is seen for the strains carrying LipR protein (Ps537 and Ps1039).

Gel retardation with partially purified LipR from *E. coli* demonstrates binding to the lipase promoter. To support the argument that LipR protein interacts with the lipase promoter, we partially purified the protein from an *E. coli* DH10B overexpressing strain and tested it in a gel retardation assay. Conditions of expression are described in Materials and Methods. SDS-PAGE analysis and peptide mass mapping allowed the identification of a 51.9-kDa protein as a product of *lipR* gene. Cell extracts from *E. coli* DH10B strains (*lipR* mutant and $lipR^+$ strains) were used for one-step purification on a heparin-Sepharose column. LipR (*E. coli* DH10B/pME6032LipR) present in a cell-free protein extract was found to bind effectively to the heparin matrix at pH 6. Peak fractions, analyzed by SDS-PAGE (with approximately 50% LipR purity), were taken to investigate their abilities to bind to radioactively labeled DNA probe $P_{lipA}199$ by gel retardation assay. $P_{lipA}199$ corresponds to the lipase promoter regulatory region from −183 to +16 (7). This shorter probe was created, as the $P_{lipA}367$ probe used initially had the tendency to form dimers (data not shown). In EMSA, we compared the binding abilities of fractions originating from $LipR^+$ and $LipR^-$ *E. coli* extracts eluted from the heparin column. Figure 6B demonstrates a band shift indicating a formation of a DNA-protein complex for a fraction containing LipR protein (lanes 2 and 3), whereas no such complex can be seen for a fraction lacking LipR (lanes 4 and 5).

DISCUSSION

The present studies show for the first time that a two-component system, named LipQ-LipR, is involved in the regulation of lipase expression. The transcription start of the lipase operon in *P. alcaligenes* is similar to the consensus of σ54-type promoters with a UAS (7). This points to a mechanism of positive control of lipase gene transcription by a regulatory protein similar to the one found for other σ54 promoters (30). In searching for such a regulatory protein, we first embarked on an attempt to complement lipase-negative mutants in *Pseudomonas alcaligenes*. However, the number of lipase-negative mutants found was far higher than expected. Most likely, many of these mutants were in fact deficient in export or protein-folding functions, as found for *P. aeruginosa* (11). In order to overcome this problem, we used a selection for positive phenotypes—the phenotype enhancement method—to search for elements that are in a direct way controlling lipase production (14). Thus, a cosmid library was introduced into *P. alcaligenes* strain Ps770, which carries a pJRDlipAB plasmid for increased lipase production. It was anticipated that the selection of lipase-hyperproducing strains would result in the identification of factors limiting lipase expression. From three independent tests with 485 strains containing the introduced cosmid library, which covers about four times the size of the chromosome, 20 were selected with hyperproduction of lipase, as judged by halo formation on a tributyrin oil plate. One of these selected 20 hyperproducing strains was identified as carrying the complete *P. alcaligenes* type II secretion pathway described earlier (14).

During fermentation experiments, due to plasmid loss, tetracycline and/or neomycin resistance for some of the 20 tested strains was gone. Therefore, the cosmids were introduced independently in strain Ps537 containing a single chromosomal lipase operon, *lipAB*. Four cosmid-containing strains with bigger halos on tributyrin oil plates were found to share an identical DNA fragment that has similarity with the family of two-component regulator systems represented by NtrBC (34). Comparison and alignment of data reveal that the first gene encoding LipQ protein is similar to that for the NtrB type of kinase and the second gene encoding LipR protein resembles that for the NtrC type of DNA binding regulator protein. In addition, relaxation of control by overexpression of the two-component system resulted in a threefold increase of lipase production in a mimicked large-scale fermentation. The enhanced lipase production fits perfectly with the copy number of pLARF3 (49) derivates in *P. alcaligenes* (two to four copies) (13).

As mentioned earlier, the LipRQ system bears resemblance to the CbrAB two-component system from *P. aeruginosa*. Importantly, CbrB and LipR show significant amino acid sequence similarities, and the same is true for CbrA and LipQ. Yet, it is interesting that in contrast to LipR, the homologous CbrB protein so far has not been shown or indicated to share the same mode of action on the lipase promoter. The CbrAB two-component system of *P. aeruginosa* was proposed to be involved in the utilization of carbon and nitrogen sources (37, 51), thus playing an important role in environmental adaptation. Still, there are no reports yet implying the direct interaction of any promoter sequences with CbrB transcriptional activator, and the histidine operon and/or arginine operon are potential candidates only. By inspecting the promoter sequences of these two operons, the probable σ54 box (−12 to −24) can be found just for the arginine operon, with no obvious σ54-type promoter for the histidine operon (51). Interestingly, existing reports indicate that CbrB appears to be important in the expression of the histidine operon in particular (37, 44, 51). In contrast to the CbrAB system, the LipQR system has not been shown to be essential in carbon-nitrogen utilization in *P. alcaligenes*, indicating that the LipQR and CbrAB regulatory systems may play different regulatory roles under

physiological conditions. Moreover, the signals that determine the CbrAB activity remain unknown, and regulation by the CbrAB system seems to be a complex mechanism that includes the regulation of several catabolic pathways. Moreover, it is not known yet whether *lipQR* genes are cotranscribed.

Our results demonstrate that the overexpression of *lipQR* up-regulates (Fig. 2) and the inactivation of *lipR* down-regulates (Fig. 5) lipase expression. Moreover, the upstream region of the lipase gene from *P. alcaligenes* possesses, at the expected distance from a promoter at −24 to −12, a UAS (TGT-N11-ACA) (3) which shows all the attributes to serve as a template for the binding of the activated LipR regulator. Thus, the involvement of LipR in the regulation of lipase expression seen from fermentation studies, coupled with the demonstrated down-regulation of lipase at the transcriptional level by inactivation of *lipR* (qRT-PCR assay) (Fig. 5B), substantiates our hypothesis and establishes LipR as a lipase regulator.

Finally, results from the band shift experiment ($P_{lipA}199$ probe) with partly purified proteins from *E. coli lipR*$^+$ versus *E. coli lipR*-negative strains demonstrate LipR protein DNA binding properties. The retardation of the DNA probe by formation of the DNA-LipR complex was present only for a protein sample containing LipR (Fig. 6B). Supporting results come also from EMSA with Ps537 and Ps1039 cell crude protein extracts (Fig. 6A, lanes 2 to 7), where more DNA formed a complex with proteins from Ps1039 than with proteins from the Ps537 strain, which is in agreement with the fact that Ps1039 overexpresses LipR. The presence of retarded bands with cell-free protein extract from the Ps1100 strain (*lipR* mutant) (Fig. 6A, lanes 8 to 10) most likely is the result of binding other protein(s). The lipase promoter sequence includes a long DNA stretch with a UAS up to the region from −12 to −24 and further downstream. Thus, proteins like σ54 protein (2, 33), another enhancer binding protein (8, 39), or an integrating host factor (16, 43, 54) could also interact with a DNA probe, resulting in a DNA-protein complex formation. Bacterial cells possess numerous two-component signal transduction systems which facilitate adaptation to environmental changes by regulation of the expression of particular genes, and it is therefore not surprising that the elucidation of regulatory mechanisms of gene expression is not always straightforward.

In conclusion, we have found and cloned a new two-component system, *lipQR*, which is involved in the regulation of lipase expression in *P. alcaligenes*, with LipQ being the putative phosphorylating component and LipR the DNA binding protein. The commercial relevance of this finding is exemplified in the hyperproduction of lipase upon overproduction of LipR.

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